A comparative study on culture conditions and routine expansion of amniotic fluid-derived mesenchymal progenitor cells

Gucciardo, L ; Ochsenbein-Kölble, N ; Ozog, Y ; Verbist, G ; Van Duppen, V ; Fryns, J P ; Lories, R ; Deprest, J

Abstract: Background: Amniotic fluid (AF) cell populations will be applied in perinatology. We aimed to test the feasibility of large-scale cell expansion. Study Methods: We determined the best out of three published expansion protocols for mesenchymal progenitors (AF samples, n = 4) in terms of self-renewal ability. Characterization was performed based on morphology, surface marker analysis, cytogenetic stability, and differentiation potential. The conditions for the best self-renewal ability were further determined in a consecutive series (n = 159). Results: The medium containing fetal bovine serum (FBS), epidermal growth factor, insulin, transferrin, and tri-iodothyronine, combined with seeding on gelatin-coated wells, best stimulated the growth of cells with mesenchymal features, as demonstrated by flow cytometry; however, only osteogenic differentiation was possible. Large-scale testing (n = 44) failed to confirm a robust self-renewal ability. Better results were obtained (n = 88) using optimized FBS or an increased initial cell density. Eventually over 81% of cultures continued growing after the initial medium change and had mesenchymal features but failed differentiation assays. Discussion: Routine in vitro expansion of AF-derived mesenchymal cells remains problematic. Despite an increase in successful cell cultures from 40 up to 80% using optimized serum and an increased cell density, eventually cells failed to demonstrate differentiation abilities. Routine isolation and expansion from unselected AF samples remains a challenge.

DOI: https://doi.org/10.1159/000354895

Original Paper

Fetal Diagnosis and Therapy

Fetal Diagn Ther 2013;34:225–235
DOI: 10.1159/000354895

Received: May 16, 2013
Accepted after revision: August 8, 2013
Published online: October 10, 2013

A Comparative Study on Culture Conditions and Routine Expansion of Amniotic Fluid-Derived Mesenchymal Progenitor Cells

L. Gucciardo[a, e] N. Ochsenbein-Kölble[a] Y. Ozog[a] G. Verbist[a] V. Van Duppen[b]
J.P. Fryns[c] R. Lories[d, f] J. Deprest[a, e]

[a]Cluster Organ Systems, [b]Stem Cells and Embryology, [d]Skeletel Biology and Engineering of the Department of Development and Regeneration and [c]Center for Human Genetics, Faculty of Medicine and Departments of [e]Obstetrics and Gynaecology and [f]Rheumatology, University Hospitals Leuven, Leuven, Belgium

Key Words
Amniotic fluid · Cells · Self-renewal and differentiation abilities · Mesenchymal features · Cell culture conditions · Characterization

Abstract
Background: Amniotic fluid (AF) cell populations will be applied in perinatology. We aimed to test the feasibility of large-scale cell expansion. ***Study Methods:*** We determined the best out of three published expansion protocols for mesenchymal progenitors (AF samples, n = 4) in terms of self-renewal ability. Characterization was performed based on morphology, surface marker analysis, cytogenetic stability, and differentiation potential. The conditions for the best self-renewal ability were further determined in a consecutive series (n = 159). ***Results:*** The medium containing fetal bovine serum (FBS), epidermal growth factor, insulin, transferrin, and tri-iodothyronine, combined with seeding on gelatin-coated wells, best stimulated the growth of cells with mesenchymal features, as demonstrated by flow cytometry; however, only osteogenic differentiation was possible. Large-scale testing (n = 44) failed to confirm a robust self-renewal ability. Better results were obtained (n = 88) using optimized FBS or an increased initial cell density. Eventually over 81% of cultures continued growing after the initial medium change and had mesenchymal features but failed differentiation assays. ***Discussion:*** Routine in vitro expansion of AF-derived mesenchymal cells remains problematic. Despite an increase in successful cell cultures from 40 up to 80% using optimized serum and an increased cell density, eventually cells failed to demonstrate differentiation abilities. Routine isolation and expansion from unselected AF samples remains a challenge.

Introduction

Mesenchymal stem cells (MSC) were first isolated from adult bone marrow [1, 2] and later from a variety of other organs. The potential of MSC to differentiate into osteoblasts, chondrocytes, adipocytes, myoblasts, neural cells, and stromal fibroblasts [3–7] and their apparently low immunogenicity after allogenic transfer make MSC a very attractive cell type for tissue repair and regeneration and other cell therapy applications [8–10]. MSC, in particular those derived from bone marrow, are already widely used clinically. They are an adjuvant to reestablish the hematopoietic system following radiation and/or chemotherapy, stimulating engraftment and preventing graft-versus-host disease [11, 12]. Coronary artery infusion of autolo-

KARGER

E-Mail karger@karger.com
www.karger.com/fdt

1015–3837/13/0344–0225$38.00/0

Jan Deprest, MD, PhD
Department of Obstetrics and Gynecology
University Hospitals Leuven, Herestraat 49
BE–3000 Leuven (Belgium)
E-Mail jan.deprest@uz.kuleuven.be

gous bone marrow-derived MSC improved cardiac function 3 months after cardiac infarction [13]. The ability of MSC to differentiate into neurons and astrocytes led to their use in the treatment of symptomatic ALS [14]. Injection of allogenic MSC has been shown to slow down the typical degradation process of lysosomal storage diseases [15]. MSC are also proposed as excellent candidate cell sources for tissue engineering biomaterials [16].

MSC also appear attractive in fetal or perinatal medicine [16, 17]. MSC isolated from first-trimester fetal blood have already been used as an allogenic graft for the treatment of children with osteogenesis imperfecta and significantly reduce bone brittleness [18, 19]. MSC derived from umbilical cord vessels have the ability to migrate in vitro towards areas of alveolar type II cell damage and to assist in cell repair probably through paracrine effects [20]. Another potential application is the transduction of MSC by a variety of vectors for their use as vehicles for either short- or long-term gene transfer [16]. Further, they could be used in tissue engineering applications for the treatment of congenital birth defects, for instance to provide alternative cell-seeded matrices to replace synthetic materials required for reconstruction of the defect [21, 22]. Experimentally, the proof of principle for this has already been provided [23–25].

In other words, the fetus may be considered as a host who is treated after birth with autologous tissue-engineered prostheses, or as a donor, providing the cellular components to process the biomaterials required after birth [21]. Several fetal tissues, including liver, bone marrow, lung, and pancreas, have been shown to contain cells with MSC characteristics, plastic properties, and a remarkable self-renewal ability [26]. Several cell types with self-renewal abilities, multilineage differentiation potential, and mesenchymal features have been successfully isolated from the fetal adnexa (membranes, placenta, and cord) [20, 27–30]. Since 2007, these cells have been classified using the consensus established by an international workshop [31]. Unfortunately, these cells only become spontaneously available at the time of birth, limiting their availability and applications for autologous treatments. However, amniotic fluid (AF) can also be retrieved prenatally with an acceptable risk, allowing successful isolation of this type of cells [32]. Amniocentesis is indeed offered in patients with structural defects to rule out genetic problems or to measure certain metabolites that correlate with the severity of the disease involved. In selected cases, one might consider harvesting a certain amount of additional AF which could be used for extracting cells. These can then be further cultured and manipulated in vitro during the remainder of pregnancy. Either during pregnancy or, more often, after birth, these cells might be used to treat the fetus or neonate [33].

Several methods for isolation of MSC have been described. They are based on selection of cells based on their physical characteristics (e.g. spindle shaped, fibroblast-like aspect) or adherence properties to plastic [29, 34, 35] or to other extracellular matrix components [27, 36]. Also various media have been used to culture and expand MSC [27, 29, 35, 36]. In this study we aimed to select an appropriate medium and culture conditions to isolate and expand cells with mesenchymal features and self-renewal and differentiation abilities derived from AF in a routine clinical setting.

Materials and Methods

Culture of Cells Derived from AF

Small-Scale Comparison of Three Culture Conditions

We initially selected 4 AF samples of male fetuses obtained during different types of fetoscopic procedures. Three AF samples were collected during laser coagulation of placental vessels in twin-twin transfusion syndrome at a mean gestational age (GA) of 19 ± 3 weeks. One sample was obtained at the time of fetal endoscopic tracheal occlusion (FETO) for a severe diaphragmatic hernia at 29 weeks and 4 days of gestation. Samples had a confirmed normal karyotype, their starting volume exceeded 20 ml, and the bacteriologic examination was negative. AF samples were centrifuged for 5 min at 400 *g*. Cells were plated in 6-well plates (Nunc, Roskilde, Denmark) and cultured on polystyrene only or fibronectin (100 ng/ml; Sigma, Bornem, Belgium) or gelatin-coated wells (Sigma) and using the following 3 media which were previously used for growing human AF-derived MSC [27, 28], amnion mesenchymal cells [37], and bone marrow-derived multipotent progenitor cells [36]:

- medium 1: M199 (Gibco, Merelbeke, Belgium) supplemented with 10% fetal bovine serum (FBS; Stem Cell Technologies, Grenoble, France), 20 μg/ml endothelial cell growth factor (ECGF; Roche Diagnostics, Vilvoorde, Belgium), and 8 U/ml heparin (Sigma);
- medium 2: DMEM-H/Ham's F12 1:1 (Sigma) supplemented with 10% FBS, 50 ng/ml epidermal growth factor (EGF), 2.5 μg/ml insulin, 5 μg/ml transferrin, and 0.1 ng/ml tri-iodothyronin (T3) (all from Sigma);
- medium 3: 60% DMEM (Gibco), 40% MCDM-201 (Sigma) supplemented with 2% FBS, 1× insulin-transferrin-selenium (Sigma), 1× chemically defined lipid concentration (Gibco), 10 ng/ml platelet-derived growth factor (PDGF; R&D, Abingdon, UK), 10 ng/ml EGF, 25 μg/ml L-ascorbic acid (Sigma), and 0.5 nmol/ml dexamethasone (Sigma).

After 4–5 days, nonadherent cells were removed and the media refreshed. When grown to subconfluence, adherent cells were detached with 0.25% trypsin-EDTA (Sigma), manually counted, and replated at 10^3 cells/cm^2 in culture flasks (T25 and T75; Falcon, Erembodegem-Aalst, Belgium). This point in time was considered as passage 0 (P0).

Human synovial-derived MSC were obtained from the synovial membrane (SM) of healthy adults as described previously [38] and cultured as control stem cells in DMEM (Gibco) supplemented with 10% FBS. All media were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Merelbeke, Belgium) and replaced every 2–3 days. All cells were cultured in duplicate in a 95% humidified atmosphere at 37°C and 5% CO_2. Self-renewal ability was evaluated through the calculation of cell doublings [formula: log (number of cells at time 1/number of cells at time 0)/log2] and the population doubling time [formula: (time 0 – time 1) × log2/log (number of cells at T1/number of cells at T0)] to determine the protocol yielding the best cell self-renewal ability.

Large-Scale (n = 159) Prospective Validation

The optimal culture condition was further used on a larger scale. During the course of the experiments, further attempts were made to increase the efficacy, such as using an optimized FBS or seeding an initial higher cell number, as detailed in Results. In total, 159 consecutive AF samples were obtained during routine clinical procedures, such as genetic amniocentesis (n = 86) and fetoscopic interventions for complicated monochorionic twin pregnancies (n = 44) and FETO procedures (n = 29). The mean GA for amniocentesis were 16.9 ± 0.3 weeks, 19.4 ± 1.5 weeks for laser coagulation, and 28.8 ± 1.2 weeks for fetuses undergoing FETO. These samples will be hereafter referred to as 'prospective samples'. The primary outcome measure was again self-renewal ability. AF-MSC were characterized after >20 cell doublings by morphology and FACS analysis and differentiation potential by immuno- and cytochemical staining. Cytogenetic stability was determined by karyotyping at prelevation and after >20 cell doublings. This characterization time point was determined to allow enough cell expansion for all of the required assays.

Flow Cytometric Analysis

After a second trypsinization (this was considered as passage 1; P1), cells were washed with phosphate-buffered saline (PBS; Gibco) containing 0.1% bovine serum albumin (BSA; Sigma). The cells cultured from the initial samples used for the culture condition comparison were incubated with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or allophycocyanin (APC)-conjugated antibodies for 30 min at 4°C or room temperature according to the manufacturer's instructions. Cells were analyzed using a Becton-Dickinson (BD, Erembodegem-Aalst, Belgium) 3-channel flow cytometer and quantification was performed using BD FACS Diva software.

For the prospective validation study, cells were analyzed using a BD 2-channel flow cytometer with FITC- and PE-conjugated antibodies. Quantification was performed using BD FACS Diva software.

We selected different surface markers based on a review of the literature [28, 29, 39]. The following antibodies against MSC markers were used: CD166 (Acris, Herford, Germany), CD105 (R&D), CD73 (BD), CD44 (BD), and CD29 (Biosource, Nivelles, Belgium). As hematopoietic stem cell markers we used: CD14 (Biosource), CD34 (BD), and CD45 (R&D). Antibodies against immunologic markers were: HLA-ABC (MHC I; BD) and HLA-DR (MHC II; BD). Unlabeled cells were used as negative controls and isotype control antibodies to quantify nonspecific binding. 7-Aminoactinomycin D (7-AAD, 1:100; BD) was added to all samples in order to exclude autofluorescence of dead cells.

Differentiation Ability

In vitro Osteogenesis

AF-derived cells as well as SM-derived MSC were replated at 2×10^4 cells/cm^2. Osteogenesis was induced with DMEM medium (10% FBS) supplemented with 10 μmol/l dexamethasone, 10 nmol/l 1α,25-dihydroxy-vitamin D_3, 50 μg/ml ascorbic acid, and 10 mmol/l β-glycerophosphate (all from Sigma). For each sample, unstimulated cells were used as controls. Alizarin red S staining was performed to identify calcified matrix. After fixing the cells in ice-cold 70% ethanol (Fluka, Bornem, Belgium), calcium precipitates were stained with 2% Alizarin red S (Sigma) in PBS. Matrix mineralization was demonstrated by the presence of a red deposition.

In vitro Adipogenesis

AF-derived cells as well as SM-derived MSC were replated at 2×10^4 cells/cm^2. Adipogenic differentiation was initiated using DMEM medium (10% FBS) supplemented with 0.5 mmol/l isobutyl-methylxanthine (IBMX), 1 μmol/l dexamethasone, 200 μmol/l indomethacin (all from Sigma), and 10 μmol/l insulin (Novo Nordisk, Brussels, Belgium). Oil red staining was performed to identify cytoplasmic lipid droplet accumulation. Cells were fixed with ice-cold methanol (Fluka) and washed with deionized water. Samples were stained with 0.5% (w/v in isopropanol) Oil red O (Sigma) and counterstained with Mayer's Hematoxylin (Fluka).

Karyotyping by G-Banding Analysis

Initial karyotyping was performed on cells from fresh AF, and later cells from cultures after 20 cell doublings were karyotyped to demonstrate cytogenetic stability.

Statistics and Ethical Considerations

Results are presented as means ± SEM. Statistical analysis was performed using Fisher's exact test, the Mann-Whitney U test, and the Kruskal-Wallis test with Statistica for Windows XP. $p < 0.05$ was considered statistically significant. This project was approved by the Ethics Committee for Clinical Studies of the University of Leuven, Belgium, and informed consent was obtained for each patient.

Results

Comparison of Culture Conditions

Cell Morphology

At the time of the first seeding, the attached AF-derived cells displayed heterogeneous sizes and morphology, with small cobblestone-like cells, larger irregular cells, and spindle-shaped cells. When the medium was refreshed 5–7 days after the first seeding, and nonadherent cells were removed, a majority of spindle-shaped cells were observed, with this phenotype becoming exclusive around confluence (fig. 1a). By around 10–15 population doublings, medium 1 on uncoated or fibronectin-coated wells, as well as medium 3, irrespective of the coating type, eventually yielded cells that could not

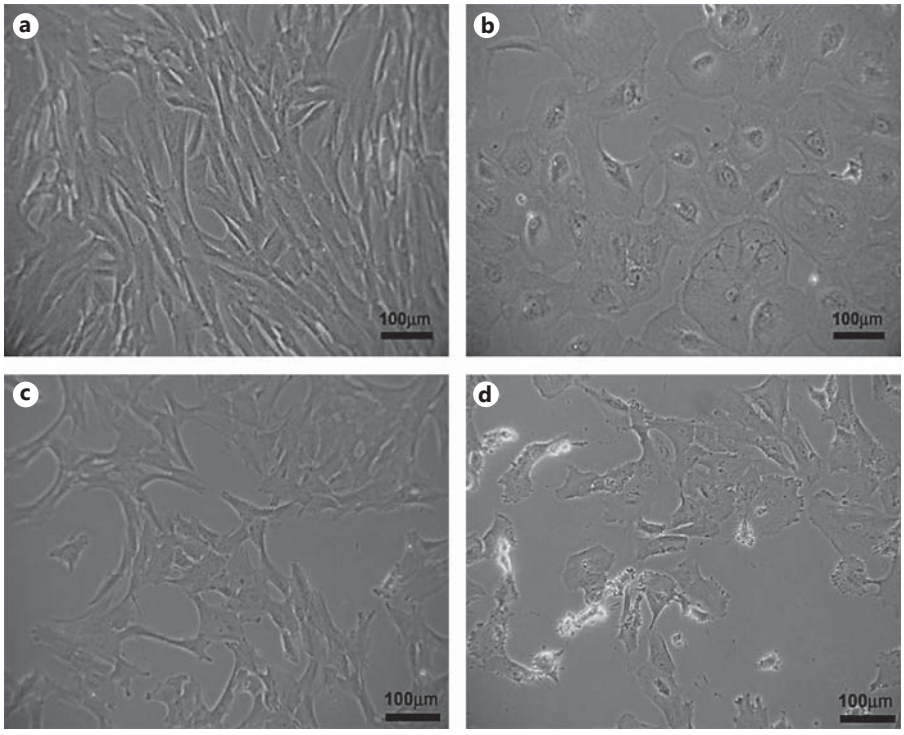

Fig. 1. AF-derived cells seeded on polystyrene with medium 2 shown at 12 population doublings (mainly spindle-shaped cells and few larger and flat cells) (**a**). When cultured in medium 3 on polystyrene, these cells (at 16 population doublings) were broader and flatter (**b**). After 28 cell doublings, AF-derived cells cultured on polystyrene in medium 2 became flatter (**c**) and showed more dying cells (**d**). Magnification ×200.

be further used in the experiment. Cells became broader and more flattened and ceased to replicate beyond 4 weeks and/or underwent cell death (fig. 1b). More precisely, this was after 97 ± 5 days and 10 ± 2 cell doublings (medium 1, fibronectin) and 14 ± 1 cell doublings (medium 1, uncoated) or 15 ± 1 cell doublings (medium 3, gelatin), 10 ± 2 cell doublings (medium 3, fibronectin), and 13 ± 1 cell doublings (medium 3, uncoated). These cells were not further sorted or tested for differentiation or karyotyped. After 20 cell doublings, cells cultured with medium 2 lost their spindle-shaped form (fig. 1c, d).

Cell Growth Kinetics

Overall, cell proliferation was faster when using medium 2 compared to either of the other two media, irrespective of the coating used (fig. 2a–c). Cell doublings with medium 2 on the 3 different coatings were comparable [33 ± 2 (gelatin), 30 ± 2 (fibronectin), and 28 ± 1 (uncoated), respectively; $p > 0.05$] at a mean of 128 ± 7 days. There were no differences in the overall cell doubling time (gelatin: 66 ± 7 h, fibronectin: 66 ± 5 h; uncoated: 87 ± 13 h; $p > 0.05$). When compared with medium 2 on gelatin, cells cultured in medium 1 on gelatin-coated wells displayed higher cell doubling times (82 ± 6 h, $p < 0.001$) and lower cell doubling numbers (26 ± 2 cell doublings, $p = 0.005$) compared to cells cultured in medium 2 on gelatin (66 ± 7 h and 33 ± 2 cell doublings, respectively). Interestingly, the mean cell doubling time for cells cultured in medium 2 was 40–60 h for the initial 20 cell doublings, and it increased to ≥94 h thereafter (table 1). A similar trend was found for cells seeded on gelatin and cultured in medium 1.

Flow Cytometry

As a control, 3 cultures of SM-derived MSC were used. Cases were AF-derived cells cultured in medium 1 and plated on gelatin (n = 8), or cultured in medium 2 and plated on gelatin (n = 6), fibronectin (n = 5), or uncoated wells (n = 6). They were characterized at a mean cell doubling of 21 ± 1. Flow cytometry analysis of all AF-derived cells stained positive for the MSC markers CD29, CD44, CD73, CD105, and CD166, but was negative for the hematopoietic stem cell markers CD14, CD34, and CD45 (fig. 3). The surface marker expression pattern was similar to the profile of human SM-derived MSC (fig. 4). However, SM-derived MSC cultures showed a significantly higher percentage of cells expressing CD105 and CD166 compared to AF-derived cells (fig. 4; $p < 0.05$). All AF-derived MSC expressed HLA-ABC (MHC I) but not HLA-DR (MHC II). This was also the case for SM-derived MSC, but more cells stained positive for MHC II

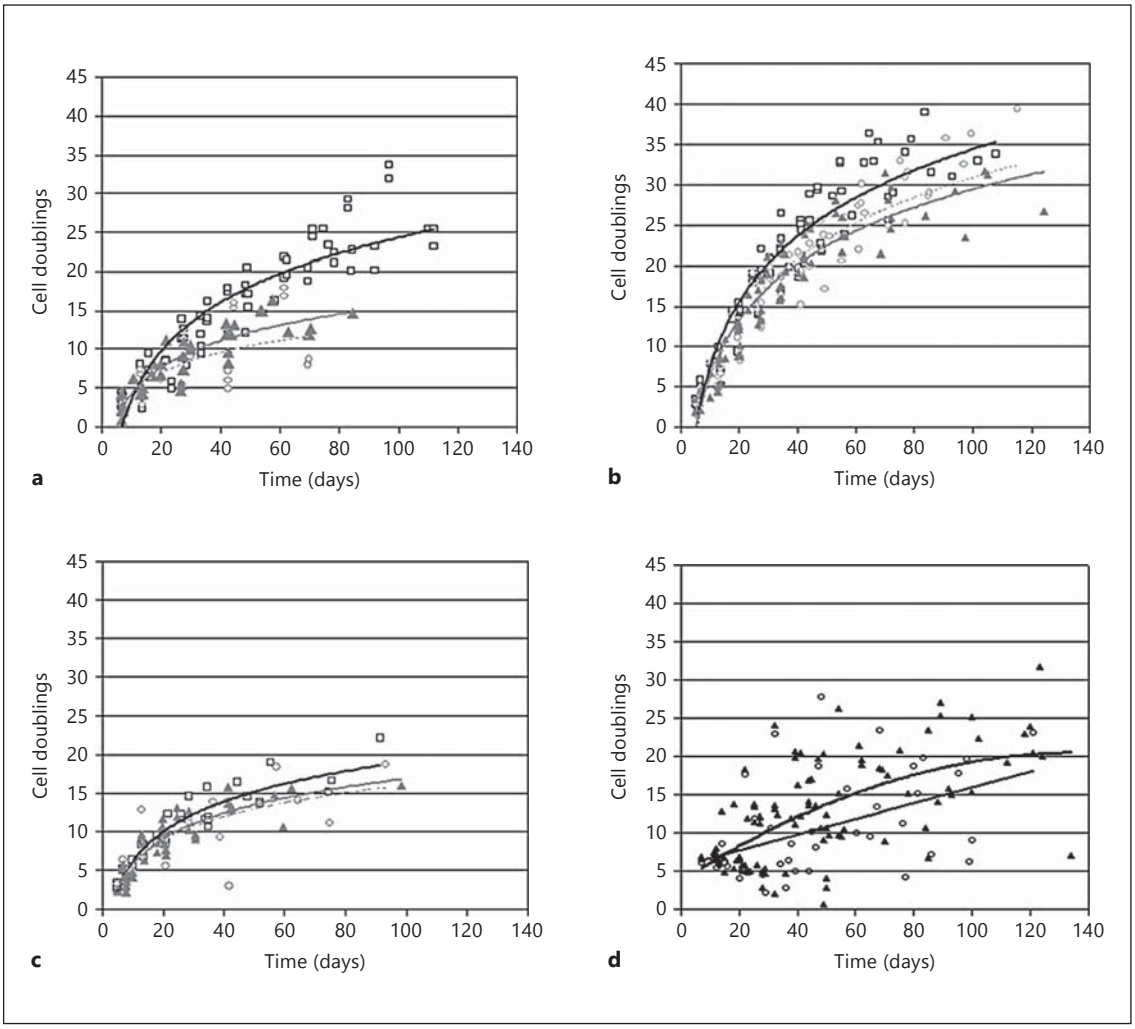

Fig. 2. Comparison of cell doublings over time, dependent on the medium and coating of culture flasks used. **a** Medium 1. **b** Medium 2. **c** Medium 3. **d** FBS vs. O-FBS. When using medium 2, the highest cell doubling number and fastest proliferation were obtained. Squares and black lines represent results for coating with gelatin, circles and dashed lines represent fibronectin, and triangles and grey lines represent polystyrene (no coating). The difference with the other two media disappeared after 130 days (**a–c**). Comparison of cell doublings over time using medium 2 with either FBS (circles and lower line) or optimized FBS (triangles and upper line). Higher cumulative cell doublings were observed with O-FBS (**d**).

($p < 0.05$). Comparing the results of the cases, AF-derived MSC cultured on gelatin in medium 1 had significantly more CD29+ cells ($p = 0.005$) than those cultured in medium 2, irrespective of the coating.

Differentiation Ability

The numbers of cell doublings prior to differentiation were comparable. In vitro adipo- and osteogenesis were evaluated. Osteogenic differentiation was effective (fig. 5). After stimulation with an osteogenic medium, SM-derived MSC showed a higher percentage of calcified cell matrices compared to AF-derived MSC seeded on gelatin

Table 1. Cell doubling times categorized by cell doublings and according to the media and coating used

	Cell doubling times (mean ± SEM), h			
	medium 1	medium 2		
Cell doublings	gelatin	gelatin	fibronectin	polystyrene
≤20	71±5	40±2	50±3	59±11
>20	115±17	97±12	94±8	147±29

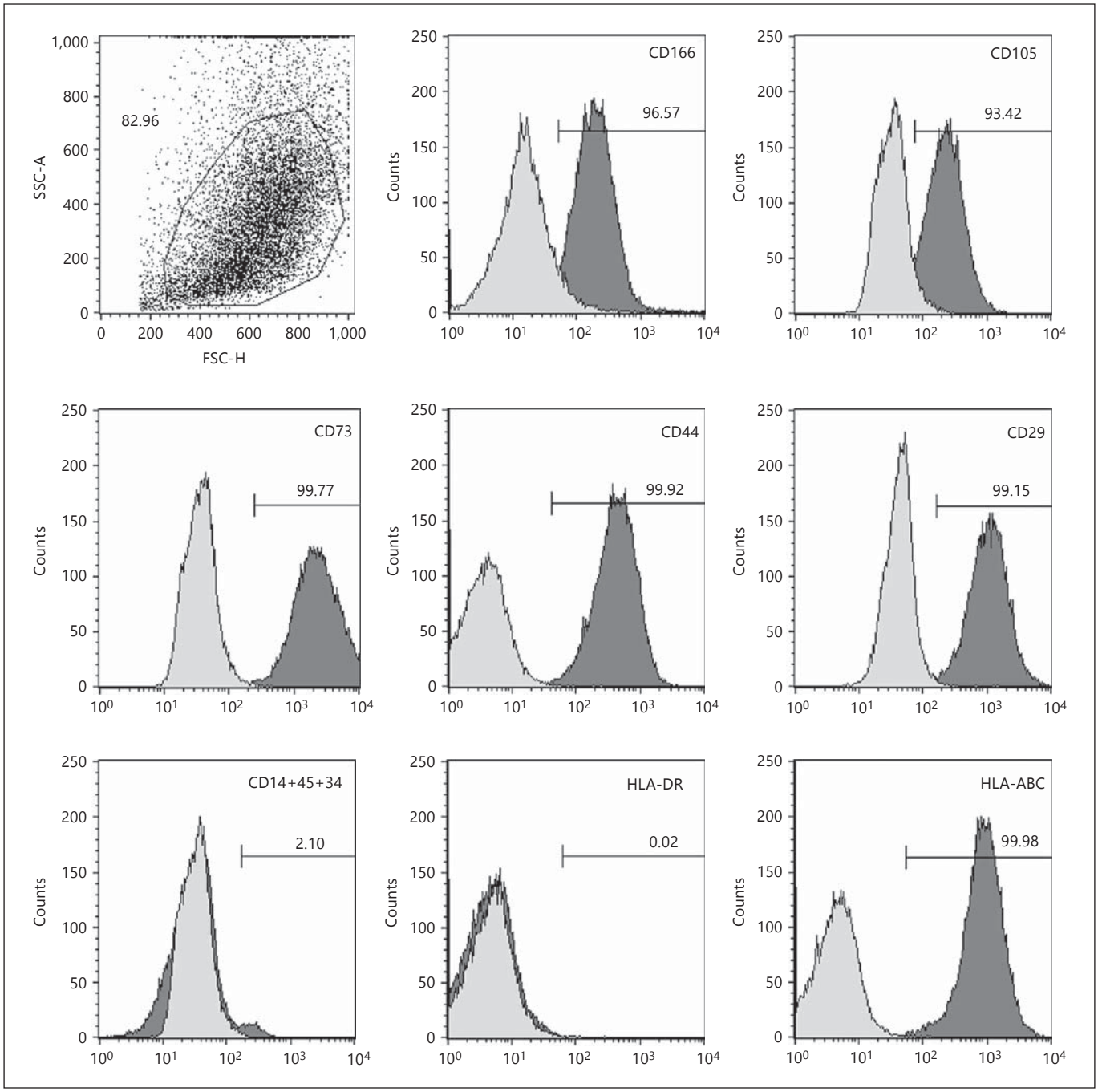

Fig. 3. Representative flow cytometry surface marker profile of AF-derived cells. Dark grey represents positively labeled cells for the given markers, and light grey represents the isotype controls.

in medium 1 ($p < 0.05$) but comparable calcification rates in relation to AF-derived MSC cultured in medium 2 in wells coated with gelatin or fibronectin or left uncoated ($p > 0.05$). Differentiation of AF-derived MSC into adipocytes was not successful.

Karyotype Using G-Banding Analysis

Cytogenetic analysis of AF cells prior to culture and from cultures using medium 1 or 2 on gelatin, fibronectin, or polystyrene showed a normal male karyotype after 21 ± 2 cell doublings.

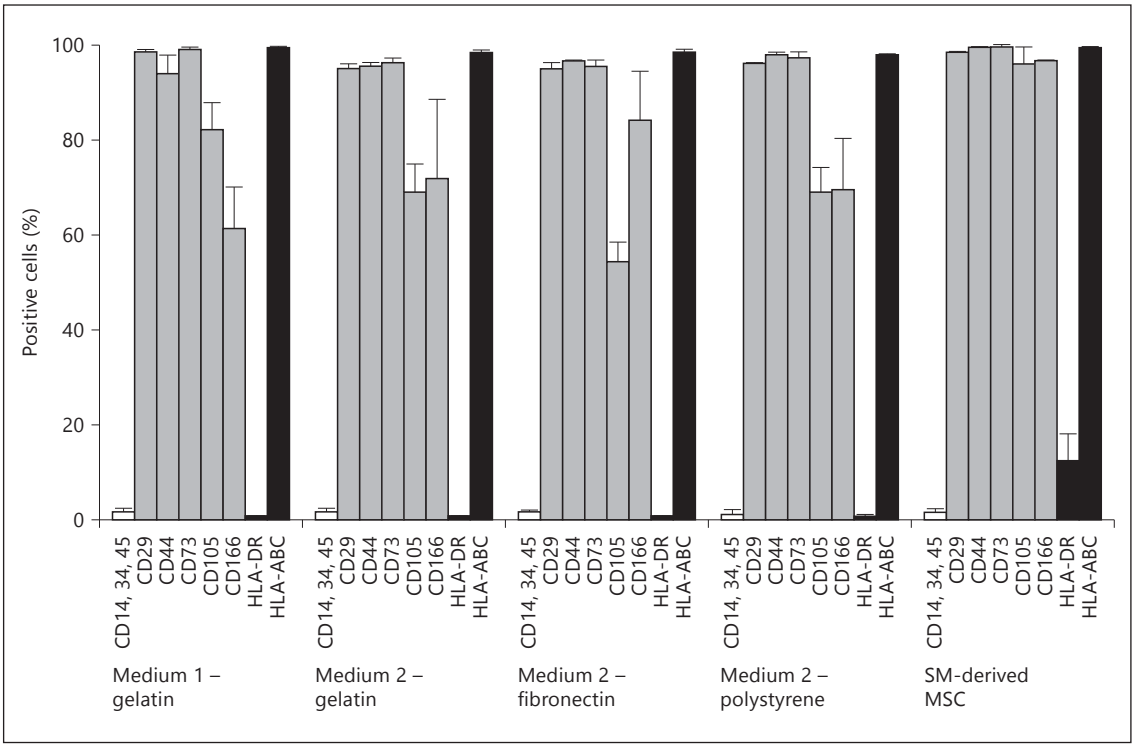

Fig. 4. Comparison of FACS surface marker profiles of AF- and SM-derived cells. Grey represents MSC markers; white represents hematopoietic markers, and black represents immunologic markers.

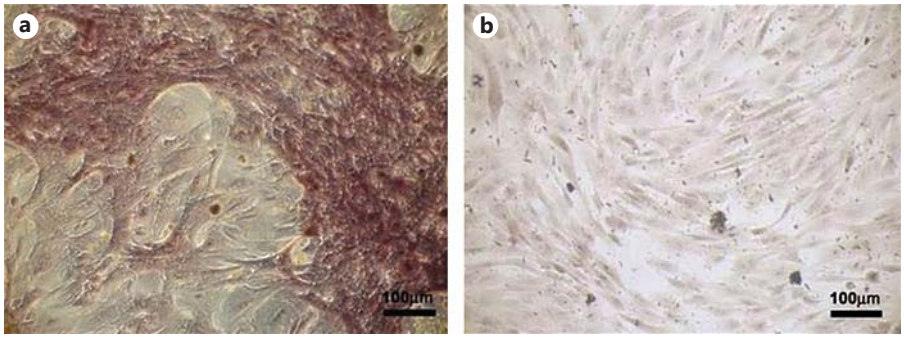

Fig. 5. Representative example of AF-derived cells isolated with medium 2. Cells were thereafter cultured at P1, after osteogenic stimulation. Alizarin red S stain shows calcified extracellular matrix (**a**). As a reference, unstimulated cells were stained in the same conditions (**b**). Magnification ×200.

Routine Expansion of Cells with Mesenchymal Features from Prospective AF Samples

Cell Morphology and Growth Kinetics

Based on the information presented above, medium 2 on gelatin-coated well plates was further used to culture prospective AF samples. In an initial 44 samples, spindle-shaped cells were now growing in only 29.5% (n = 13) of cases. In the other 70.5% (n = 31), when the medium was refreshed at 5–7 days and nonadherent cells were removed, an absence of cell growth with a majority of larger irregular cells was observed. In the remainder, where spindle-shaped cells were growing until subconfluence, those eventually became broader and more flattened and ceased to replicate after trypsinization and reseeding in 53.8% of cases (n = 7). In 6 samples where cells continued to grow, the mean cumulative cell doubling was 12.1 ± 1.7, with a mean cell doubling time of 171.5 ± 22.6 h. This low number of growing cells, combined with a very low self-renewal ability, were judged to be an insufficient result and an obstacle for further cell characterization and

Table 2. Self-renewal ability according to the serum used for culture of amniotic cells obtained from pregnancies prior to or beyond 20 weeks of gestation

		FBS	O-FBS
Cell growth rate, %	Overall	40.9	48.9
	≤20 weeks	40.3	50.8
	>20 weeks	42.3	46.1
Cell growth rate >P0, %	Overall	69.4	90.7[a]
	≤20 weeks	64.0	90.0[a]
	>20 weeks	81.8	91.7
Cumulative PD	Overall	10.0±1.5	15.9±1.5[b]
	≤20 weeks	8.8±2.0	15.1±1.4[b]
	>20 weeks	11.8±2.2	17.9±3.7
Maximum number of passages	Overall	1.4±0.2	2.7±0.3[b]
	≤20 weeks	1.1±0.3	2.7±0.3[b]
	>20 weeks	1.9±0.4	2.8±0.6
PDT, h	Overall	168.2±27.0	170.6±24.7
	≤20 weeks	211.7±53.7	117.8±74.5
	>20 weeks	126.3±16.7	120.6±24.7

Differences between media were only significant for the cell growth rate after the first trypsinization (P0), and population doubling (PD) time (PDT) in the cells obtained prior to or at 20 weeks. [a] Significant difference, $p < 0.05$ (Fisher's exact test). [b] Significant difference, $p < 0.05$ (Mann-Whitney U test).

differentiation. For that reason, we questioned whether the medium used was consistently identical. Upon detailed revision, one component of the medium was FBS which, during the course of the experiments, originated from different lots. FBS batch differences have been recognized as a cause of discrepant results [40]. Actually, a better self-renewal ability has been reported when one uses FBS that is optimized for expansion of human MSC (O-FBS) [28, 34].

From that point on, we prospectively compared 88 cultures with the initially used FBS (Gibco) parallel to using O-FBS (Stem Cell Technologies, Grenoble, France). Nonadherent cells were removed when the medium was refreshed 5–7 days after the first seeding. Two different cell morphologies were observed according to the serum used to reconstitute the medium. Cells cultured with FBS were irregular, with a large broad cytoplasm. Adherent cells cultured with O-FBS were exclusively spindle shaped. The overall cell growth rates (defined as the percentage of cell cultures continuing after the first medium refresh) were not different (table 2). The medium with O-FBS yielded a higher number, showing cell growth after the first trypsinization (defined as P0), reaching higher cumulative population doublings (fig. 2d). Secondary analysis by GA upon harvesting the initial AF sample showed better self-renewal properties prior to, rather than after, 20 weeks, as earlier described [27] (table 2).

Flow Cytometry

Eighteen routine AF samples from the 'prospective group' were characterized at a mean population doubling of 12.7 ± 0.8. Again cells were positive for MSC markers CD29, CD44, CD73, CD105, and CD166 and negative for hematopoietic stem cell markers CD14, CD34, and CD45. Also AF-derived MSC showed a high percentage of positive cells for HLA-ABC (MHC I) combined with a low number of cells staining positive for HLA-DR (MHC II). There were no significant differences in surface markers between cells cultured with FBS and cells cultured with O-FBS.

Differentiation Ability and Karyotype

The prospective samples did not reach the required population doubling to determine their differentiation potential and their karyotype stability.

Additional Improvement in Culture Conditions Using an Increased Initial Cell Density

A small percentage of the AF cell population had self-renewal and differentiation abilities. Therefore, we decided to increase the cell density to 10^5 cells/cm^2, the initial cell density seeded in parallel cultures using 27 prospective AF samples. The overall cell growth rates observed with both sera (FBS vs. O-FBS) were not significantly different (80.8 vs. 81.4%). These overall cell growth rates using a cell density of 10^5 cells/cm^2 were significantly higher than the cell growth rates observed in previous experiments using a density of 10^3 cells/cm^2 combined with FBS or O-FBS (41.3 and 48.3%, respectively). Cells were characterized using the same protocol as for the small-scale comparison. There were no significant differences in surface markers. Cells cultured with both sera were positive for MSC markers CD29, CD44, CD73, CD105, and CD166 with a high percentage of positive cells for HLA-ABC (MHC I) and a low number of cells staining positive for HLA-DR (MHC II). These cells were negative for hematopoietic stem cell markers CD14, CD34, and CD45. Cytogenetic analysis of AF cells prior to culturing and from cultures showed a normal karyotype. Differentiation assays were performed but failed to show differentiation abilities.

Conclusion

Our study evaluated different culture conditions for isolation and characterization of AF-derived cells with mesenchymal features and self-renewal and differentiation abilities. These culture conditions were previously reported for successful isolation of MSC and AF-derived cells. Although in vitro expansion of AF cells is possible, routine expansion in a prospective cohort in a setting mimicking what eventually may happen clinically was problematic. Though using different media and seeding of cells on gelatin-coated wells provided better results, the success rate (overall cell growth rate) remained below 50% with a low initial cell density (10^3 cells/cm^2) protocol which was, again, considered clinically insufficient. Increasing the cell density to $10^5/cm^2$ at the first seeding resulted in a success rate of 81%, but differentiation assays failed to demonstrate multilineage potential. Therefore, culture of these cells remains a challenge, indicating the need for better culture media and conditions, which may be based on better characterization and selection of the initial cell population [29, 39].

In our initial experiments, with media containing either 2 or 10% FBS, the cell growth was similar until approximately 10 cell doublings. Thereafter, AF-derived cells grew better with a medium containing EGF, insulin, transferrin, and T3 besides 10% FBS (medium 2), and cell doubling times were independent of surface coatings. The amount of FBS used in different studies about AF-MSC [27–30, 39, 41], amnion mesenchymal cells [37], and bone marrow-derived multipotent progenitor cells [36] varied between 2 and 20%. Given these differences, the quoted self-renewal ability may not be comparable between different papers. Such differences are a common observation in publications about AF or placenta-derived cells with mesenchymal features and self-renewal and differentiation abilities. It reflects the heterogeneous methodologies used to isolate and to expand these cells and a lack of consensus about classification of the isolated cell population. An important improvement occurred in 2008 with the first international workshop about placenta-derived stem cells. A consensual classification was published to standardize the methodologies used for placenta-derived MSC isolation. The minimal criteria for defining placenta-derived MSC were as follows: adherence to plastic; formation of fibroblast colony-forming units; a specific pattern of surface antigen expression (positivity for CD73, CD90, and CD105 and negativity for CD14, CD34, CD45, and HLA-DR), and differentiation potential toward one or more lineages, including osteogenic, adipogenic, chondrogenic, and vascular/endothelial. Surprisingly, this consensus did not included AF-derived cells [31]. Recently, De Coppi et al. [39] illustrated the heterogeneity of AF cell populations. They isolated a subgroup of AF-derived cells based on the expression of the surface antigen c-kit (CD117) and proposed to call these AF stem cells. They observed a remarkable cell self-renewal ability which had never been reported by others in the field, with up to >250 population doublings obtained with a culture medium containing 15% of FBS. This is far superior to observations made in studies using adult MSC expanded up to 30 population doublings in approximately 130–200 days [42, 43]. Interestingly, Kim et al. [41] described that AF-MSC were able to undergo 66 population doublings over a time period of 8 months. In that study approximately 40 cell doublings could be reached after 130 days of cell culture, implying a possible higher proliferative potential of fetal MSC compared to adult MSC, as described previously [26, 28]. Kim et al. [41] focused on the molecular profile and characterization of AF-derived stem cells, with less or no information about the number of AF samples, the cell density used to start the initial culture, and the observed cell growth rate compared to the number of processed samples. In the present study, we decided to highlight the influence of different culture conditions on the selection of a cell line with consistent self-renewal ability and differentiation properties in an initial small number of AF samples. Thereafter, we tested our observations on a larger scale, a preliminary step that we considered an essential hurdle to overcome before further investigating this cell source for perinatal applications.

The differentiation assays were set up after 20 cell doublings. Osteogenic differentiation was possible but adipogenesis was not, although in previous studies successful differentiation was tested at earlier cell doublings showing bilineage [27, 28], trilineage [29, 30], or even multilineage [39, 41] potential. Selection of a specific cell type through our isolation and expansion protocol may be a potential cause for this observed restriction in differentiation ability. Bossolasco et al. [30] suggested that their limited differentiation potential towards mesenchymal lineages could be due to cell heterogeneity with a high percentage of epithelial cells. During culture, bone marrow stromal cells progressively lose their adipogenic and chondrogenic differentiation potential. The adipogenic potential is apparently the first to be repressed, whereas the osteogenic and chondrogenic potentials proceed together, branching only very late, with the loss of chondro-

genic differentiation ability after 22 cell doublings [44]. Other authors have reported similar findings [42, 45].

In the initial series, the culture condition with the best results was medium 2 combined with the gelatin coating. Our initially promising results were not confirmed when expanding on a larger scale in unselected AF samples, so that it was not possible to perform characterization, differentiation, and karyotyping as scheduled. Therefore, we made two adjustments to our culture conditions. We tested another FBS optimized for AF-MSC isolation and improved our results. We finally tested a high initial cell density protocol with optimal cell self-renewal ability results. Cells isolated with these two culture condition improvements showed mesenchymal surface markers, but differentiation assays failed to show multilineage potential. Testing optimized FBS and the high initial cell density protocol with media 1 and 3 would certainly have been interesting, but we did not. The reason is that we consider the methodology described by De Coppi et al. [39] to be the best available option for isolation and expansion of AF-derived stem cells. The disadvantages of commercial ready-to-use medium are the lack of composition control and that it may be difficult to use in clinical conditions (as most of the time it still contains bovine serum). The optimal AF-derived cell culture conditions need to be as simple as possible, with a minimal amount of compounds, and made without xenogeneic material. Such conditions should ideally be associated with monoclonal cell selection and expansion. Therefore, we have since moved on to protocols that expand clones of cells based on certain characteristics, such as c-kit expression [39].

A limitation of the present study is that the effects of cytokines on cell proliferation and identification of pluripotency markers (Oct-4, Nanog, Sox2, SSEA3, and SSEA4) were not analyzed. This was not routinely done at the moment this study was designed. The cell population we obtained in our study, though it showed some self-renewal ability and mesenchymal features (surface markers and unilineage differentiation ability), was distinct from amniocytes. However, those cells do not match the definition of an MSC or an AF stem cell. In summary, the conditions we used may have identified conditions where AF-derived mesenchymal progenitor cells could be cultured with reasonable efficacy, though the success rate and cell characteristics are not sufficient for a clinically applicable harvesting program. However, we have not given up on the use of AF as a cell source and have moved on to more effective protocols [46].

Acknowledgements

This work was supported by grants from the Swiss National Science Foundation, the European Commission in its 6th Framework Programme (MEST CT2005 019707; EuroSTEC, LSHC-CT-2006-037409), and the Instituut voor Wetenschap en Technologie (I.W.T.). Leonardo Gucciardo has been supported by the Klinisch Onderzoeksfonds of the University Hospitals Leuven. Jan Deprest is a clinical researcher for the Fonds Wetenschappelijk Onderzoek-Vlaanderen.

References

1 Friedenstein AJ, Chailakhjan RK, Lalykina KS: The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 1970; 3:393–403.
2 Friedenstein AJ, Gorskaja JF, Kulagina NN: Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 1976;4:267–274.
3 Pittenger MF, Mackay AM, Beck SC, et al: Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–147.
4 Prockop DJ: Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997;276:71–74.
5 Minguell JJ, Erices A, Conget P: Mesenchymal stem cells. Exp Biol Med (Maywood) 2001;226:507–520.
6 Le Blanc K, Pittenger M: Mesenchymal stem cells: progress toward promise. Cytotherapy 2005;7:36–45.
7 Benvenuti S, Saccardi R, Luciani P, et al: Neuronal differentiation of human mesenchymal stem cells: changes in the expression of the Alzheimer's disease-related gene seladin-1. Exp Cell Res 2006;312:2592–2604.
8 Aggarwal S, Pittenger MF: Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005;105:1815–1822.
9 Le Blanc K, Ringden O: Immunobiology of human mesenchymal stem cells and future use in hematopoietic stem cell transplantation. Biol Blood Marrow Transplant 2005;11: 321–334.
10 Le Blanc K, Ringden O: Mesenchymal stem cells: properties and role in clinical bone marrow transplantation. Curr Opin Immunol 2006;18:586–591.
11 Bhatia M, Wang JC, Kapp U, et al: Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc Natl Acad Sci USA 1997;94:5320–5325.
12 Osawa M, Hanada K, Hamada H, et al: Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 1996;273:242–245.
13 Chen SL, Fang WW, Ye F, et al: Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Am J Cardiol 2004;94:92–95.
14 Mazzini L, Mareschi K, Ferrero I, et al: Autologous mesenchymal stem cells: clinical applications in amyotrophic lateral sclerosis. Neurol Res 2006;28:523–526.
15 Koc ON, Day J, Nieder M, et al: Allogeneic mesenchymal stem cell infusion for treatment of metachromatic leukodystrophy (MLD) and Hurler syndrome (MPS-IH). Bone Marrow Transplant 2002;30:215–222.
16 Javazon EH, Beggs KJ, Flake AW: Mesenchymal stem cells: paradoxes of passaging. Exp Hematol 2004;32:414–425.

17 Siegel N, Rosner M, Hanneder M, et al: Human amniotic fluid stem cells: a new perspective. Amino Acids 2008;35:291–293.
18 Guillot PV, Abass O, Bassett JH, et al: Intrauterine transplantation of human fetal mesenchymal stem cells from first-trimester blood repairs bone and reduces fractures in osteogenesis imperfecta mice. Blood 2008; 111:1717–1725.
19 Horwitz EM, Prockop DJ, Fitzpatrick LA, et al: Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 1999;5:309–313.
20 Montemurro T, Andriolo G, Montelatici E, et al: Differentiation and migration properties of human fetal umbilical cord perivascular cells: potential for lung repair. J Cell Mol Med 2011;15:796–808.
21 Turner CG, Fauza DO: Fetal tissue engineering. Clin Perinatol 2009;36:473–488, xii.
22 Gucciardo L, Lories R, Ochsenbein-Kolble N, et al: Fetal mesenchymal stem cells: isolation, properties and potential use in perinatology and regenerative medicine. BJOG 2009;116: 166–172.
23 Fuchs JR, Kaviani A, Oh JT, et al: Diaphragmatic reconstruction with autologous tendon engineered from mesenchymal amniocytes. J Pediatr Surg 2004;39:834–838, discussion 834–838.
24 Kunisaki SM, Fuchs JR, Kaviani A, et al: Diaphragmatic repair through fetal tissue engineering: a comparison between mesenchymal amniocyte- and myoblast-based constructs. J Pediatr Surg 2006;41:34–39, discussion 34–39.
25 Fauza DO, Fishman SJ, Mehegan K, et al: Videofetoscopically assisted fetal tissue engineering: bladder augmentation. J Pediatr Surg 1998;33:7–12.
26 O'Donoghue K, Fisk NM: Fetal stem cells. Best Pract Res Clin Obstet Gynaecol 2004;18: 853–875.
27 In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, et al: Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. Stem Cells 2004;22:1338–1345.
28 In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, et al: Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. Blood 2003;102:1548–1549.
29 Tsai MS, Lee JL, Chang YJ, et al: Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol. Hum Reprod 2004;19:1450–1456.
30 Bossolasco P, Montemurro T, Cova L, et al: Molecular and phenotypic characterization of human amniotic fluid cells and their differentiation potential. Cell Res 2006;16:329–336.
31 Parolini O, Alviano F, Bagnara GP, et al: Concise review: isolation and characterization of cells from human term placenta – outcome of the first international Workshop on Placenta Derived Stem Cells. Stem Cells 2008;26:300–311.
32 Abdulrazzak H, De Coppi P, Guillot PV: Therapeutic potential of amniotic fluid stem cells. Curr Stem Cell Res Ther 2013;8:117–124.
33 Trohatou O, Anagnou NP, Roubelakis MG: Human amniotic fluid stem cells as an attractive tool for clinical applications. Curr Stem Cell Res Ther 2013;8:125–132.
34 Campagnoli C, Roberts IA, Kumar S, et al: Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood 2001;98:2396–2402.
35 Portmann-Lanz CB, Schoeberlein A, Huber A, et al: Placental mesenchymal stem cells as potential autologous graft for pre- and perinatal neuroregeneration. Am J Obstet Gynecol 2006;194:664–673.
36 Reyes M, Lund T, Lenvik T, et al: Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood 2001;98:2615–2625.
37 Ochsenbein-Kolble N, Bilic G, Hall H, et al: Inducing proliferation of human amnion epithelial and mesenchymal cells for prospective engineering of membrane repair. J Perinat Med 2003;31:287–294.
38 De Bari C, Dell'Accio F, Tylzanowski P, et al: Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum 2001;44:1928–1942.
39 De Coppi P, Bartsch G Jr, Siddiqui MM, et al: Isolation of amniotic stem cell lines with potential for therapy. Nat Biotechnol 2007;25: 100–106.
40 van der Valk J, Mellor D, Brands R, et al: The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. Toxicol In Vitro 2004;18:1–12.
41 Kim J, Lee Y, Kim H, et al: Human amniotic fluid-derived stem cells have characteristics of multipotent stem cells. Cell Prolif 2007;40: 75–90.
42 Baxter MA, Wynn RF, Jowitt SN, et al: Study of telomere length reveals rapid aging of human marrow stromal cells following in vitro expansion. Stem Cells 2004;22:675–682.
43 Bonab MM, Alimoghaddam K, Talebian F, et al: Aging of mesenchymal stem cell in vitro. BMC Cell Biol 2006;7:14.
44 Muraglia A, Cancedda R, Quarto R: Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci 2000;113:1161–1166.
45 Digirolamo CM, Stokes D, Colter D, et al: Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. Br J Haematol 1999;107:275–281.
46 Zia S, Toelen J, da Cunha MM, et al: Routine clonal expansion of mesenchymal stem cells derived from amniotic fluid for perinatal applications. Prenat Diagn 2013;23:1–8. doi: 10.1002/pd.4162. [Epub ahead of print]